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CONDITIONS NECESSARY FOR TRANSFORMATION TO PROTOTROPHY AND TO THE ABILITY TO SYNTHESIZE POLYGLUTAMIC ACID IN BACILLUS LICHENIFORMIS

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CONDITIONS NECESSARY FOR TRANSFORMATION TO PROTOTROPHY AND TO THE ABILITY TO SYNTHESIZE POLYGLUTAMIC ACID IN *BACILLUS LICHENIFORMIS*

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ABSTRACT

This report presents studies on the growth conditions necessary for transformation to prototrophy of 14 auxotrophs of B. licheniformis. The unexpected finding of different growth requirements by each auxotroph for the development of transformable cells is discussed. Under optimum growth conditions for a serine-deficient mutant, transformation frequencies of 0.1 per cent were obtained. A defined medium for tube transformation of competent cells is described.

In addition, this report presents evidence for the transformation of three non-encapsulated mutants of B. licheniformis for the ability to synthesize polyglutamic acid (capsular material).

I. INTRODUCTION

Bacillus licheniformis strain 9945A synthesizes large amounts of polyglutamic acid (capsular material) during growth in a chemically defined medium.[1] We have been studying the mechanism of polyglutamic acid synthesis by *B. licheniformis*.[1-3] Genetic and biochemical studies of factors affecting polyglutamic acid synthesis by encapsulated and non-encapsulated mutants of this organism will be of great help in further elucidating the mechanism of polyglutamic acid synthesis.

Transformation of *B. licheniformis* auxotrophs had not been reported until Gwinn and Thorne[4] were able to transform to prototrophy three of a series of 28 auxotrophs of *B. licheniformis*.

This report presents studies on the growth conditions necessary for transformation to prototrophy of each of the 14 mutants we have tested. The finding of different growth requirements by each auxotroph for the development of transformable cells is discussed. This report also presents evidence for the transformation of three non-encapsulated mutants of *B. licheniformis* for the ability to synthesize polyglutamic acid (capsular material). A chemically defined medium for transformation of *B. licheniformis* in liquid suspensions is described.

II. MATERIALS AND METHODS

A. ORGANISMS

Bacillus licheniformis strain 9945A was used throughout as the donor of wild-type DNA and for obtaining auxotrophic mutants and non-encapsulated mutants after irradiation with ultraviolet light.[5] This strain produces large quantities of capsular material (L- and D-polyglutamic acid) when grown on minimal agar plates[4] or on medium E.[1] The non-encapsulated mutants (capsule$^-$) did not produce capsular material in any media tested including supplemented minimal or medium E agar plates. (Under similar conditions encapsulated strains produced large amounts of capsular material).

B. STOCK CULTURES

Spores of wild-type and mutant strains were prepared in potato broth[6] and kept at 4°C. Cells were grown from spore inoculum (1×10^6 spores per milliliter) in 50 milliliters of the desired medium in 500-milliliter flasks. The flasks were shaken at 37°C on a reciprocating shaker set at 100 excursions per minute (five-centimeter stroke) for the desired length of time.

C. MEDIA AND CULTURAL CONDITIONS

Unless otherwise specified the cells were grown in the following modifications of minimal medium designated as NBSG medium: $(NH_4)_2SO_4$, 2 grams; K_2HPO_4, 14 grams; KH_2PO_4, 6 grams; Na citrate·$2H_2O$, 1 gram, $MgSO_4 \cdot 7H_2O$, 0.2 gram; $FeCl_3 \cdot 6H_2O$, 0.04 gram; $MnSO_4 \cdot H_2O$, 0.002 gram; nutrient broth, 8 grams; glycerol, 5 grams; (added aseptically) and triple-distilled water to one liter, at pH 7.0.

Transformants were scored on medium E or on minimal agar plates. Minimal agar plates consisted of the same medium described above except that nutrient broth was omitted, glucose was substituted for glycerol, and one gram of L-glutamic acid and 15 grams of agar were added aseptically per liter of medium.

D. DESOXYRIBONUCLEIC ACID (DNA)

DNA was isolated by Marmur's procedure[7] from lysozyme lysate of 10-hour-old cells grown in NBSG. The treatment with ribonuclease and the precipitation of DNA with isopropyl alcohol were omitted. The DNA was determined by the method of Burton[8] and it was stored in 2 M NaCl at 4°C.

E. TRANSFORMATION METHODS

The mutants were tested for transforming ability at 37°C, either by the agar plate technique or by tube transformation technique in liquid suspensions. Plate transformation consisted of spreading together 0.1 ml of cells (grown in NBSG medium for any desired length of time) and 0.1 ml of DNA (40 micrograms of DNA per plate) on minimal or medium E agar plates. When the cells were diluted before plating, the diluent used was minimal medium with 1×10^{-3} M $CaCl_2$ added aseptically. Control plates included one with cells alone and one with cells, DNA, and desoxyribonuclease (40 micrograms of 1 x crystallized product of Worthington Biochemical Corp., Freehold, N.J.). The plates were incubated for two days. The tube transformation method in liquid suspensions was done in cotton-plugged test tubes (150 by 15 mm) in a final volume of one milliliter. The cells were grown in modified NBSG for the desired length of time, washed twice, and diluted with the transformation medium to the desired cell concentration. As a rule 0.1 ml of cells (3×10^8 to 6×10^8 cells), 0.1 ml of DNA in 2 M NaCl (40 micrograms), and 0.8 ml of transformation medium were placed in a test tube. The tubes were slanted on a rack and shaken on a reciprocating shaker for three to four hours at 37°C. The samples were exposed to 40 micrograms of desoxyribonuclease per milliliter for 15 minutes and then diluted in minimal medium with 1×10^{-3} M $CaCl_2$ added aseptically and plated on minimal agar or medium E plates.

III. RESULTS

A. GROWTH STUDIES ON THE APPEARANCE OF TRANSFORMABLE CULTURES

We were unable to transform auxotrophs of B. licheniformis when we used the routine procedures described for B. subtilis, strain 168,[9] for Hemophilus influenza,[10-11] and for Diplococcus pneumoneae,[12] although many modifications of these methods were tested. Difco's Penassay broth, tryptose broth, and brain heart infusion were also tested for growth of transformable cells of the serine auxotroph and no significant number of transformable cells was obtained. We also failed to develop a chemically defined medium for growing competent cells of various auxotrophs with either a spore or a cell inoculum.

We then tested cell cultures of 14 mutants grown in NBSG as described under Section II. The cells were tested directly by the plate transformation method at two-hour intervals from 0 to 54 hours of growth. Transformation was obtained with only four of these 14 mutants at different periods of incubation. When varying concentrations of glycerol were tested all of the 14 mutants were transformed to prototrophy (Table I). The optimum concentration of glycerol required may vary for each auxotroph. Also, the time of incubation in NBSG required for development of competence varied for each auxotroph. The cells of these mutants did not become significantly competent on the plates, but competent cells transformed well on the plates when plated with DNA. (Other auxotrophs such as M-28[4] can develop competence on minimal agar plates.) A small variation from the optimum glycerol concentration resulted in loss of the ability to transform. In all cases addition of desoxyribonuclease to the plates resulted in total loss of transformants.

Addition of 1×10^{-3} M $CaCl_2$ to NBSG medium resulted in a several-fold increase in the number of transformants of most auxotrophs tested. In all subsequent experiments 1×10^{-3} M $CaCl_2$ was added aseptically to NBSG.

We then tested the effect of several other components of NBSG on the transformability of cells of a serine auxotroph (M5V-3). Table II shows some of these results. The serine auxotroph transformed at a frequency of 0.11 per cent when grown for 36 to 42 hours in NBSG using 0.8 per cent nutrient broth, 1.5 per cent glycerol, 0.3 per cent sodium citrate, and 0.2 per cent $(NH_4)_2SO_4$. The cells were diluted to the desired cell concentration with minimal medium (1×10^{-3} M $CaCl_2$ added aseptically) and then were tested by plate transformation. Although not shown in Table II, the concentration of nutrient broth was also varied. When 0.4 per cent and 0.6 per cent nutrient broth were used no significant transformation of cells of various ages was obtained with this serine auxotroph. These concentrations of glycerol, sodium citrate, and $(NH_4)_2SO_4$ were found to be optimal; varying the concentration of any of these resulted in a decrease in transformation frequency. The concentrations of glycerol used also affected the age and the length of time the cultures remained transformable. When glucose was substituted for glycerol in this medium, cells could not be transformed.

TABLE I. MUTANTS OF B. LICHENIFORMIS 9945A TRANSFORMED TO PROTOTROPHY[a]

Mutant	Requirement	Glycerol Concentration, per cent	Optimum Time of Incubation for Competence, hr	Plate Transformants per 3×10^8 Recipient Cells
M1	Thiamine	0.30	14	34
M1R-1	Thiamine	0.30	13	91
M2R-1	Lysine	0.40	20	11
M4R-1	Arginine	0.40	18	174
M5	Serine	0.50	17	49
M5V-3	Serine	0.50	17	58
M8	Methionine	0.50	27	18
M8R-1	Methionine	0.35	30	52
M11	Methionine	0.50	22	60
M12	Unidentified	0.75	23	106
M14	Adenine	0.50	18	57
M17	Adenine	1.00	25	17
M18	Arginine	1.00	26	220
M19	Arginine	0.50	18	25

a. Each of the mutants was tested for transformation on minimal agar plates with DNA isolated from wild-type B. licheniformis. Cultures of recipient cells were grown for the number of hours indicated in NBSG under the conditions described in Section II.

TABLE II. EFFECT OF MEDIUM COMPONENTS ON THE COMPETENCE OF M5V-3 CELLS (SERINE$^-$)[a]

Concentration of Medium Components				
Glycerol, Per Cent	Sodium Citrate, per cent	$(NH_4)_2SO_4$, per cent	Optimum Time of Incubation for Competence, hr	Plate Transformants per 6×10^8 Recipient Cells
0.2	0.1	0.2	-	0
0.5	0.1	0.2	15	2.2×10^1
1.0	0.1	0.2	32	3.8×10^2
1.25	0.1	0.2	32-34	6.9×10^2
1.5	0.1	0.2	32-42	3.4×10^5
2.0	0.1	0.2	38-48	1.4×10^2
1.5	0.2	0.2	36-42	4.5×10^5
1.5	0.3	0.2	36-42	6.7×10^5
1.5	0.5	0.2	36-42	1.2×10^5
1.5	0.3	0	36-42	3.0×10^2
1.5	0.3	0.1	36-47	1.3×10^5
1.5	0.3	0.3	36-40	6.2×10^5
1.5	0.3	0.5	32-39	1.0×10^4

a. Other medium components at regular concentration in NBSG with 1×10^{-3} M $CaCl_2$ added aseptically. The recipient cells were diluted with minimal medium (1×10^{-3} M $CaCl_2$ added aseptically) and varying cell concentrations were tested by plate transformation.

Similar studies were done with a non-encapsulated, thiamine-requiring mutant (M1R-1). These cells transformed at a frequency of 0.0001 per cent when grown in specifically modified NBSG using 0.6 to 0.8 per cent nutrient broth, 0.3 per cent glycerol, 0.2 per cent sodium citrate and 0.2 per cent $(NH_4)_2SO_4$ and the plate transformation method. Cell cultures became competent after 14 hours of incubation and remained competent for about an hour. It was found later that cells of this thiamine-deficient auxotroph transformed at frequencies of 0.001 per cent when grown in NBSG (as described above) with added 3×10^{-4} M $MnSO_4$ and 0.15 M NaCl. The transformation frequencies were improved about 1000-fold over those obtained with unmodified NBSG but were still low compared with the serine auxotroph. It is significant to point out that a complex growth medium (NBSG) specifically modified for each mutant was the only successful means found that gave significant numbers of transformable cells of the 14 auxotrophs studied.

Efforts to transfer the cells after various times of incubation from NBSG to other complex media or to chemically defined growth media for further development of competence have failed.

In all cases the transformable cultures were in the late stationary phase of growth, since all the mutants attained maximal growth in modified NBSG after eight to ten hours of incubation. Total growth in NBSG or modified NBSG was about 3×10^9 to 6×10^9 cells per milliliter and was maintained for more than 48 hours. All of the mutants gave less than one per cent sporulation in this medium.

B. TRANSFORMATION IN LIQUID SUSPENSIONS

Several chemically defined media were tested for optimum tube transformation. Poor results were obtained with the transformation medium described by Anagnostopoulus and Spizizen.[9] A modification of this transformation medium, designated here as B.L. transformation medium, supported transformation of the auxotrophs tested. B.L. transformation medium consists of minimal medium with 0.5 per cent glucose, 0.01 per cent acid-hydrolyzed casein (vitamin-free), 5 micrograms of L-tryptophan, a minimal amount of the growth requirement of the mutant being tested, 5×10^{-3} M $MgSO_4$, 1×10^{-3} M $CaCl_2$, 9×10^{-4} M $MnSO_4$, and 0.2 M NaCl, at pH 7.0. Competent cells of a serine-requiring and a thiamine-requiring auxotroph required NaCl and two divalent cations for optimum transformation in B.L. medium. Table III shows some of these results, using a serine-deficient auxotroph. Maximum transformation frequencies of 0.11 per cent were obtained with this auxotroph when 0.1 to 0.4 M NaCl was added. A decrease or increase from these NaCl concentrations resulted in poor transformation. The two-cation requirement was satisfied by a combination of Mn^{++} and Mg^{++} or Mn^{++} and Ca^{++}, at the concentrations given in Table III.

TABLE III. EFFECT OF METAL CATIONS ON TRANSFORMATION OF M5V-3 (SERINE$^-$) IN LIQUID SUSPENSIONS[a/]

Metal Cations Studied in B.L. Medium				
NaCl	$MnSO_4$	$CaCl_2$	$MgSO_4$	Transformants per 6×10^8 Recipient Cells
2×10^{-1} M	9×10^{-4} M	1×10^{-3} M	5×10^{-3} M	
+	+	+	+	5.8×10^5
+	+	+	-	6.7×10^5
+	+	-	+	5.2×10^5
+	+	-	-	2.7×10^5
+	-	+	+	1.7×10^5
+	-	+	-	2.5×10^4
+	-	-	+	1.1×10^5
+	-	-	-	5.0×10^1
-	+	+	+	2.5×10^4

a. M5V-3 (serine$^-$) cells were grown for 39 hours in modified NBSG. The cells were washed twice and tested by tube transformation as described in the text. The tubes were shaken for four hours and the transformants were scored on minimal agar plates with 50 micrograms desoxyribonuclease added.

A combination of Mg^{++} and Ca^{++} without Mn^{++} was not as satisfactory. A combination of all three cations gave more consistent results with the mutants tested and therefore all three cations in addition to 0.2 M NaCl were used routinely in B.L. medium. Similar requirements were found for tube transformation of the thiamine-deficient auxotroph (M1R-1). This auxotroph transformed at frequencies of 0.001 per cent by tube transformation.

Tube transformation was relatively low during the first hour of incubation in B.L. transformation medium, but transformation was optimal after four hours of incubation. Similar results were obtained when the DNA was added at the start of the four-hour incubation period or when it was added 90 minutes before the end of the four-hour incubation period. Therefore, as a rule, the DNA was added at the start of the period. No significant growth of the cells occurred in this medium during the incubation period.

The optimum incubation time and metal ion concentration in B.L. transformation medium may vary with each mutant. Optimum conditions for growth and tube transformation of the other auxotrophs shown in Table I are under study.

C. TRANSFORMATION OF THE ABILITY TO SYNTHESIZE POLYGLUTAMIC ACID (CAPSULAR MATERIAL)

Since no specific method is available for selecting encapsulated transformants from the non-encapsulated recipients, only double transformants to prototrophy and capsulation can be scored. Therefore, high frequencies of transformation with non-encapsulated auxotrophs are required. As the transformation frequency to prototrophy was increased, the number of double transformants to prototrophy and capsule$^+$ also increased. A double mutant thiamine$^-$, capsule$^-$ (M1R-1) has been used in most of these studies. Figure 1 shows that double transformants, thiamine$^+$, capsule$^+$, are easily distinguished from single transformants, thiamine$^+$, capsule$^-$, when plated on minimal agar plates.

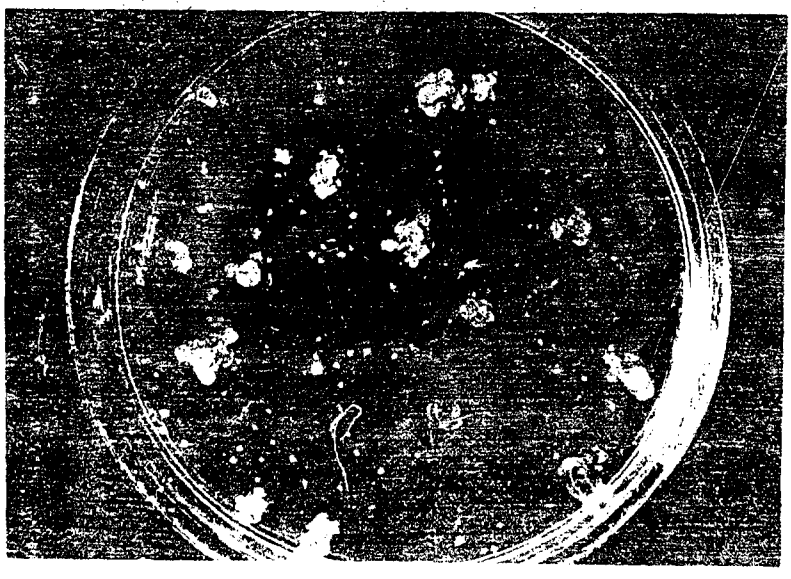

Figure 1. Encapsulated and Non-Encapsulated Transformants of *B. licheniformis* M1R-1 (Thiamine$^-$, Capsule$^-$) on Minimal Agar Plates.

Table IV shows transformation of this mutant, using DNA from several sources. When wild-type 9945A DNA or DNA extracted from a double transformant (thiamine$^+$, capsule$^+$) was used, about five per cent of the total prototrophs were also capsulated. When DNA extracted from a non-encapsulated transformant to prototrophy (thiamine$^+$, capsule$^-$) was used all the prototrophs were capsule$^-$ regardless of the amount of DNA used. DNA extracted from some other capsule$^-$ mutants of B. licheniformis transformed this mutant to capsule$^+$; others did not. This indicated that some of our non-encapsulated mutants mutated at different points in the chromosome. Homologous DNA gave no transformation. Also, addition of desoxyribonuclease to the samples resulted in total loss of transformants.

TABLE IV. TRANSFORMATION OF THE ABILITY TO SYNTHESIZE POLYGLUTAMIC ACID (CAPSULAR MATERIAL) TO M1R-1 (THIAMINE$^-$, CAPSULE$^-$)[a/]

DNA Source	Single Transformants to Thiamine$^+$	Double Transformants to Thiamine$^+$, Capsule$^+$
Wild-type 9945A	502	18
M1R-1 (thiamine$^+$, capsule$^+$)	480	23
M1R-1 (thiamine$^+$, capsule$^-$)	463	0
M1R-1 (thiamine$^-$, capsule$^-$)	0	0
9945A-1 (capsule$^-$)	280	0
9945A-4 (capsule$^-$)	310	14

a. M1R-1 (thiamine$^-$, capsule$^-$) cells were grown in NBSG medium with 0.3 per cent glycerol, 0.2 per cent sodium citrate, and 1×10^{-3} M $CaCl_2$ for 14½ hours. 3×10^8 cells were tested for transformation on minimal agar plates with 50 micrograms of the desired DNA per plate.

The ability to synthesize capsular material was also transformed to an arginine$^-$, capsule$^-$ mutant and to a methionine$^-$, capsule$^-$ mutant. Other non-encapsulated mutants are under investigation.

IV. DISCUSSION

We have been studying the mechanism of polyglutamic acid synthesis (capsular material) by Bacillus licheniformis.[1-3] In an effort to elucidate the mechanism of polypeptide synthesis we became interested in developing a system for the transformation of B. licheniformis as a tool in our biochemical studies with encapsulated and non-encapsulated mutants of B. licheniformis.

We were unable to transform auxotrophs of B. licheniformis when we used routine procedures described for Bacillus subtilis, 168,[9] for Hemophillus influenzae,[10,11] and for Diplococcus pneumoneae,[12] although many modifications of these methods were tested. Efforts to develop a defined medium for growing transformable cultures also failed. It was an unexpected finding that the use of a complex medium (NBSG) specifically modified for each auxotroph tested was the only successful means found that gave significant numbers of transformable cells of all the 14 auxotrophs studied.

In these studies with a serine-requiring auxotroph and a thiamine-requiring auxotroph it was found that varying the concentration of the various medium components from those found to be optimal for these auxotrophs resulted in a decrease in transformation frequencies.

We do not know if these special growth media reflect the selection of a more transformable cell line during growth of each mutant or whether they are necessary for getting the mutants to a specific physiological state of growth. The requirement of a specifically modified medium for optimum transformation of each auxotroph studied may account for the different transformation frequencies found among different auxotrophs of a given strain of B. subtilis and other organisms (as well as those of different isolates of the same auxotroph) when the different auxotrophs of a given organism are tested under the same set of conditions.

A defined medium for transformation of B. licheniformis in liquid suspensions is described. Competent cells of several auxotrophs of B. licheniformis required NaCl, Mn^{++}, and either Ca^{++} or Mg^{++} for optimum transformation, and the requirements for optimum transformation may vary with each auxotroph of B. licheniformis. The possible roles of these metals on DNA uptake and integration may be explained by Barnhart and Herriott[13] and Young and Spizizen[14] in recent findings and discussions of some of the factors influencing the uptake of DNA by competent cells.

This report also presents evidence for the transformation of the ability to synthesize capsular material, namely, polyglutamic acid in three non-encapsulated mutants of B. licheniformis. We have several different non-encapsulated mutants and we plan to do genetic and biochemical studies with them. These studies, in addition to our work on polyglutamic acid synthesis by cell-free extracts of B. licheniformis, may help to elucidate the mechanism of polyglutamic acid synthesis.[3]

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